FINAL REPORT

PROTOCOL CODE: FB 702 **THEMATICS:** 418/238/03

TITLE: APPLICATION OF HYALOFILL-F ON FULL- THICKNESS WOUNDS AS A PRE TREATMENT TO IMPROVE THE TAKE OF LASERSKIN AUTOGRAFTS IN A CONTROLLED PORCINE MODEL.

Principal Investigator: H.A.NAVSARIA

Collaborators: EMILIO BIGON
MASSIMO DONÀ
DANIELE PRESSATO
MANUELA DUCA
ELISABETTA DE GASPERI
DIEGO GUIDOLIN

Note: this document is composed of 22 pages including figures.

The experiment described in this report was carried out under my supervision according to the methods stated therein and the report accurately reflects the results obtained.

Dr Alessandra Pavesio
Study Director, Fidia Advanced Biopolymers

* * *

This report is the result of a study conducted to the best of my scientific knowledge and is a faithful representation of the data obtained.

Dr H.A. Navsaria - QMWC, UK
Principal Investigator

01.07.98
Date

INTRODUCTION

The take of cultured keratinocytes on full thickness wounds is poor and the resulting epidermis is fragile and prone to blistering. Pre-treatment of the wound bed with dermis significantly improves both the take rate and the quality of the resulting epidermis. Both the extracellular matrix proteins and the mesenchymal cells have been reported to play a critical role in the preparation of the wound bed. Epithelial-mesenchymal interactions are necessary for the normal development of skin. It has been shown that connective tissue components influence the growth and differentiation of epidermal cells in embryos and to a lesser extent in adult animals. Conversely, ectoderm is necessary for the development of dermis from mesenchyme. Furthermore, the breakthrough by Rheinwald and Green of serial cultivation of keratinocytes into confluent sheets was achieved, not only by the use of mitogens in the culture medium, but also by the use of a fibroblast feeder layer. Although keratinocytes can be cultured without mitogens or a fibroblast feeder layer, the Green method remains the most suitable in providing sheets of keratinocytes for clinical use. In view of the likely importance of a mesenchymal influence on keratinocyte growth after grafting, current interest is directed at providing a dermal foundation containing fibroblasts for cultured autologous grafts (CAK) when attempting to resurface full-thickness wounds.

There is evidence in the biomedical literature to suggest that Hyaluronic Acid (HA) also called Hyaluronate is intrinsically involved in the process of wound repair and that, when exogenously added, may serve a beneficial role in the acceleration or modification of the wound healing process. The biological role of hyaluronate is best understood in the process of morphogenetic cell movements, where an extracellular matrix abundant in this large, hydrophilic molecule becomes permissive for the rapid movement of cells to new sites in tissue. It also plays a positive role in regenerative and repair processes by facilitating the movement of inflammatory, mesenchymal and epithelial cells to the site of tissue repair. This process results in improved vascularisation and a favourable bed for keratinocyte grafting.

The physical properties of hyaluronate can be markedly modified by esterification, which allows processing of material into films, powders, microspheres and various fibrous structures including woven and non woven gauzes and ropes. Hyalofill-F^{TM} is a 75% benzyl esterified derivative of HA and has generally been used as a wound dressing.

AIM

The aim of this study is to test *in vivo* Hyalofill-F as a dressing for pre-treating the wound bed prior to the application of Laserskin Autograft on full-thickness wounds in a porcine model to improve the percentage of reepithelialisation.

MATERIALS AND METHODS

TEST MATERIAL

COMMON NAME	Hyalofill-F
PRODUCT CODE	346101F
TRADE NAME	Hyalofill-F
SUPPLIER	Fidia Advanced Biopolymers
LOT NUMBER	0.106.00
EXPIRY DATE	May 1999
STORAGE CONDITIONS	room temperature
PRECAUTIONS	none

ADMINISTRATION:

- **PREPARATIONS OF TEST MATERIAL FOR TREATMENT:** The test material is supplied in a 5 x 5 cm dimension sterilized by gamma-ray irradiation.
- **ADMINISTRATION ROUTE:** topical
- **JUSTIFICATION FOR CHOICE OF ROUTE:** as applied to treat cutaneous injuries
- **INTENDED DOSE:** 5x5 cm to completely cover a 4 cm in diameter wound area.
- **FREQUENCY AND DURATION OF ADMINISTRATION:**

a) one application at time of the surgery followed at day 7 by Laserskin Autograft application (group 1)

b) one application at time of the surgery and at days: 7, 14, 21, 28 (group 2).

EXPERIMENTAL MODEL

SPECIES AND STRAIN: Yucatan Miniature swine - Micropig YU

SUPPLIER: Charles-River (Lecco, Italy)
JUSTIFICATION FOR SELECTION OF SPECIES: this animal model is widely used for studies on skin repair due to a large surface area and the similarity of the skin with human skin from an anatomical point of view .
NUMBER OF ANIMALS: 6
SEX: male
WEIGHT: 25-30 Kg
AGE: appx 1 year
MICROBIOLOGICAL CLASSIFICATION: Conventional animals, housed under controlled conditions. Periodic examinations of health is performed using microbiological, parasitological and serological techniques aimed at identifying undesirable and pathogenic organisms.
IDENTIFICATION: Each animal, on arrival to laboratory, was assigned an identifying code tattooed on the ear. The animals were housed individually in pens.

ANIMAL CARE

Part referred to the pig "Condizioni di stabulazione standard adottate nei laboratori di ricerca Fidia" pag. 2.
HOUSING: Peripheral Animal Care Unit at FIDIA RESEARCH LABORATORIES. On arrival and until completion of experimentation, animals were housed individually.

EXPERIMENTAL DESIGN

The study is divided into two phases:
- first phase necessary to prepare keratinocytes cultures using Laserskin as support material;
- second phase to evaluate if the pre-treatment of the wound bed with Hyalofill F, improves the take of keratinocytes and the quality of the resulting epidermis

Anaesthesia and post surgery treatments
In all phases animals were anaesthetised by intramuscular injection of 8 ml of a xilazine (20 mg/ml) and ketamine (50 mg/ml) solution in a 1:2 ratio. Anaesthesia was maintained by further intravenous injections of 5-10 mg/Kg of a 60 mg/ml pentobarbitone solution, when required.
Fidia SOP AC 190, that refers to post operatory treatments, was completely followed in the case of the wound creation on the animals.

Harvesting of split skin graft and keratinocyte cultures

For this experiment 6 mini-pig were used, general anaesthesia induced and hair on one flank was removed by means of an electrical shaver.

The flank was cleaned with Iodine solution followed by Chlorhexidine. A split- skin graft with an approximately 6x6 cm area and a 0.25 mm thickness, was harvested using an electric dermatome.

The wound created was a partial thickness wound which would heal spontaneously by secondary healing. This is the normal way of harvesting skin in humans.

The wound was dressed with Jelonet (paraffin gauze) followed by a gauze dressing and covered with Opsite (occlusive dressing). The donor site was completely healed by 7-10 days.

Autologous keratinocyte cultures were established from the split skin graft by trypsin digestion of epidermis, the primary cultures were then plated onto Laserskin membrane in the presence of a feeder layer of non proliferating murine fibroblasts. After seven days Laserskin autograft was ready to be grafted onto the individual animals.

Induction of skin injuries and treatment

About 10 days after the skin harvesting, general anaesthesia was induced in each animal, the flanks were completely depilated, and the areas cleaned and disinfected respectively with a Ibiscrub solution (surgical detergent) and with iodinated solution.

A maximum of 6 (3 per flank) 4 cm in diameter full-thickness circular wounds were made so as to completely remove dermal and epidermal tissues, leaving intact the underlying muscular fascia (fig1A).

A PTFE chamber was then placed directly onto the wound bed using the edges of the skin to bury the flanges of the chamber subcutaneously. The chamber was secured to the surrounding skin with 2/0 silk sutures. The test material Hyalofill (fig 2) was applied on appropriate wounds and left for one week as in fig 1B after which keratinocytes on Laserskin were applied (fig 1C).

The 6 wounds constitute six test sites for the 4 planned groups of the study, each site was in turn treated in such a way to fulfil the scheme below:

EXPERIMENTAL SCHEME:

GROUPS	number of site of test	TREATMENT	DOSE	KERATINOCYTES (°)	DRESSING (°°)	Route of administr.
1.	11	Hyalofill F (*)	5x5 cm	Laserskin Autograft	Jelonet	topical
2.	11	Hyalofill F (**)	5x5 cm	-	Jelonet	
3.	7	-	-	Laserskin Autograft	Jelonet	
4.	7	-	-	-	Jelonet	

* single application only on the day of surgery
** application on the first day of surgery and after at day: 7, 14, 21, 28
° one week after the application of Hyalofill F
°° application on the day of surgery and after at day: 7, 14, 21, 28

All 4 groups were present on individual animals to account for the individual animal variation.
On the day of surgery after planned treatment, the animals were wrapped around with an elasticated bandage. A rigid lightweight jacket, lined with foam and strapped around the trunk of the animal, to protect the wounds.
At the planned time points: 7, 14, 21, 28 days, animals were anaesthetised, sites were treated according to the allocated groups, each wound was biopsied and photographed and at the end the animals was dressed as described previously.
The experiment lasted for a maximum of 6-8 weeks in total.

IMAGE ANALYSIS

Percentage reepithelialisation was calculated using computer image analysis. Briefly the image of the wound is screened onto a visual display unit and the area with epithelial cover is mapped into the computer. Similarly the area of the chamber is also mapped. The percentage of epithelial cover for each wound, at each time point is thus calculated.

HISTOLOGY AND IMMUNOCYTOCHEMISTRY

After sampling the biopsies were divided in two parts: one part fixed in formalin for "hematoxilin-eosine" and "Mallory trichromate" staining. Methods used were as per standard protocol.

The other part was snap frozen in liquid nitrogen used for immunocytochemistry analysis. 5um frozen sections are cut on a cryostat and stained with the appropriate antibodies using a three layer staining method.

ELECTRON MICROSCOPY

Performed as per standard protocol.

RESULTS

% reepithelialisation

From the graph reported in page 12 it can be observed that 70% of the wound is epithelialised in the group 1 treated with Hyalofill and Laserskin as compared to 27% in the group 3 treated with laserskin alone at the 4 week time point. This result is significant ($p < 0.001$). Epithelial cover in the group 2 treated with Hyalofill alone is the same as group 1 and there is no significant difference between these two groups. In group 4 there is hardly any epithelium present at all.

Clinical Appearance

Within this model in those wounds in which transplanted keratinocytes have taken, a few islands of immature epithelium are observed within the chamber at 2 weeks after grafting, which enlarge, opacify and become more stable over the weeks. Epithelial islands are very difficult to observe at very early time points. At 1 week after grafting the membrane was still present on the wound surface. It became brittle and separated when the dressings were removed at 2 or 3 weeks after grafting to reveal any epithelial take. There was more epidermal cover in wounds treated with Hyalofill (fig 3A and 3C) as compared to group 3 (fig 3B). The new epidermis was initially transparent but but 10-14 days after grafting, it became opaque in a confluent manner. At 4 weeks the epidermis was quite durable and able to with stand mild abrasion. Epidermal cover was absent at the periphery of the wounds adjacent to the walls of the skin graft chambers, where a sero-purulent exudate covered a raw granulating area. The epidermis in group 3 was clinically fragile. Areas of hyperpigmentation were present in all the wounds.

Histology

1 week after grafting the wounds with Hyalofill (fig 4A) a better organised wound bed can be observed as compared to granulation tissue alone (fig 4B). The most striking feature is the strong angiogenic response present in the wounds treated with Hyalofill and the blood vessels are perpendicular to the surface (directed angiogenesis). This angigenic process appears to be controlled in the presence of epidermis in the later time points. In groups 1 and 2 the new epidermis was irregularly acanthotic in the 1 week grafts, but it gradualy matured and became less hyperplastic, so that by 3-4 weeks the epidermis was only slightly thicker than that of normal porcine skin. Rete ridges are present in group 1 (fig 4A) with a normally stratified epidermis and surface cornification. In group 3 the dermo-epidermal junction is flat (fig 4B) devoid of rete ridges. The collagen appears to be less mature compared with that in the Hyalofill treated wounds at equivalent time points.

Electron Micrographs

In the 4 week grafts of group 1 the basal keratinocytes had a normal content of tonofilaments and fewer visible mitochondria. Desmosomes are present, as were interdigitations between adjacent cells. The dermal surface of the basal keratinocytes was branching and undulating. The lamina densa was continuous along the dermo epidermal junction and anchoring fibrils were plentiful (fig 5A). Hemidesmosomes are completely differentiated in the group 1 wounds with a basal plate whereas they appear to be incomplete in group 3 with a dense appearance in the basal laminae (fig 5B). Collagen bundles are present in abundance in group 1 wounds and sparse in the group 3 wounds. Interestingly the collagen bundles are parallel to the epidermis (fig 5C) in group 1 a sign of normal remodelling of the neo-dermis which has implications on scarless wound healing.

CONCLUSIONS

The epidermal cover was significantly greater ($p<0.001$) when Laserskins were applied to a Hyalofill treated wound bed as compared to a granulating wound surface (Fig. A). Furthermore the epidermis on the Hyalofill treated wounds was qualitatively superior, clinically durable (Fig. 3A) and histologically resembled normal pig skin in having rête ridges, normal stratification and surface cornification (Fig. 4C). Ultrastructurally the basal keratinocytes and the basement membrane zone in the 4 week wounds were identical to those in normal skin (Fig. 5). In contrast the epidermis on wounds in

REFERENCES

1. Navsaria HA, Kangesu T, Manek S, Green CJ, Leigh IM. An animal model to study the significance of dermis for grafting cultured keratinocytes on full thickness wounds.(1994) Burns. 20; S57-S60.

2. Navsaria HA, Myers SR, Leigh IM, McKay I. Culturing skin *in vitro* for wound therapy; (1995) Trends Biotech. 15; 91-100.

3. Myers SR, Navsaria HA, Sanders R, Leigh IM. Transplantation of keratinocytes in the treatment of wounds. (1995) Am J Surg. 170; 75-83.

4. West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. (1991) Science. 228; 1324-6.

5. Davidson JM, Beccaro M, Pressato D, Dona M, Pavesio A. Biological response of experimental cutaneous wounds in the pig to hyaluron ester biomterials.(1994) Proceed Euro Soc Biomat; 44-51.

6. Gallico GG. Biologic skin substututes. (1990) Cli Plast Surg. 17; 519-26

7. Meckenzie IC, Hill MW. Connective tissue influences on patterns of epithelial architecture and keratinization in skin and oral mucosa of adult mouse. (1984) Cell Tissue Res. 235, 551-9.

8. Cuono C, Langdom R, McGuire J. Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury. (1986) Lancet 1, 1123-4.

9. Kangesu T, Navsaria HA, Manek S, et al. A porcine model using skin graft chambers for studies on cultured keratinocytes. (1993) Br J Plast Surg. 46, 393-400.

% Re-epithellalisation of full-thickness wound in a porcine model